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Insulin effect on the cell cycle: Analysis of the kinetics of growth parameters in confluent chick cells

(insulin and serum/mitogenicity/flow microfluorometry/fibroblasts)

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ABSTRACT **Several techniques, including flow microfluorometry, were utilized to study the effect of insulin on the growth of cultured cells. It was demonstrated that chick fibroblasts can be stimulated to synthesize DNA and undergo mitosis after insulin addition. The kinetics of the cell movement through the cell cycle as well as the length of the cycle itself, however, were distinctly different in insulin- and serum-treated cultures. The insulin-treated cells had a shorter G_1, an extended S, and a much extended G_2 residence time compared to cells treated with serum. A model of growth regulation which includes both primary cultures and cell lines is proposed.**

That hormones regulate the growth of animal cells in culture has been reported widely (1–3). Insulin is the one hormone that has been frequently studied in both avian and mammalian systems. The history of the involvement of insulin as a growth-stimulating factor for cells in culture, especially chick cells, is almost as old as that of tissue culture itself. Temin (4) has proposed that insulin acts as one of the serum factors that is necessary for the growth of chick embryo fibroblasts. Several peptides with insulin-like activity and growth-stimulating potential for chick cells have since been isolated which give credence to this view (5–9).

Other reports, however, suggest that insulin, by itself, is either incapable of triggering the initiation of DNA synthesis, or that it can only stimulate the initiation of DNA synthesis, but not the completion of DNA synthesis and mitosis (10–12). Nevertheless, in most cases after the addition of insulin, a substantial increase was reported in the acid-precipitable material labeled with [^{3}H]thymidine (11, 12). The reason behind the reported lack of concomitant increase in cell number has not been clear. In addition, insulin does not stimulate DNA synthesis in certain cell lines of mouse embryo fibroblasts (13). In short, the total picture of insulin action on growth regulation has been confusing and at times contradictory (4, 6, 10, 11).

Previous studies on the response to insulin have primarily followed cell growth by measuring either [^{3}H]thymidine incorporation into DNA, or increases in the mitotic index and cell number. The fate of cells which may have been stimulated to grow by insulin has never been clearly defined. The technique of flow microfluorometry (FMF; ref. 14), developed for the detection of DNA content per cell, makes it possible to monitor cells as they progress through the cell cycle. The possible role of insulin as a growth-stimulating factor was determined by detailed analysis of the kinetic responses as indicated by various growth parameters which included the cell cycle sequence of confluent embryo fibroblasts after the addition of insulin or chick serum. The differences observed between insulin- and serum-treated cultures, and some of the contradictory results reported in the literature, may be understood in the light of the present work.

Abbreviation: FMF, flow microfluorometry.

MATERIALS AND METHODS

Materials. Chicken eggs were obtained from H & N, Inc., Redmond, Wash.; culture plates from Becton, Dickinson and Co.; Medium 199 from Grand Island Biological Co.; calf serum and chick serum from Microbiological Associates; bovine pancreatic insulin (25.7 U.S.P. units/mg), colcemid, and calf thymus DNA from Calbiochem; tryptose phosphate broth from Difco Lab; mithramycin from Nathan Belcher of Pfizer Chemical Co.; and [5-*methyl*-^{3}H]thymidine (20 Ci/mmol), from New England Nuclear.

Preparation of Cell Culture. Primary chick fibroblast cultures were prepared as described by Rein and Rubin (15). Secondary cultures were seeded at 1 to 1.1 × 10^6 cells per 35 mm plate (16). The cells were grown for 32–36 hr to confluency at a density of 1.7 to 1.9 × 10^6 cells per plate in 2.5 ml of Medium 199 containing 2% tryptose phosphate broth, 2% calf serum, and 1% chick serum. The medium was removed and cultures were washed three times with warm saline buffer. The cultures were incubated further in 2 ml of serum-free Medium 199 for 12 hr or more, at which time the medium was changed to fresh Medium 199 containing serum or insulin. The times of changing the medium and addition of serum or insulin were staggered after serum deprivation so that growth parameters could be measured all at one time. Alternatively, factors were added at 12 hr after serum deprivation and growth parameters were measured at times indicated in the figures thereafter. No significant difference in growth response was observed between the two methods.

Cell Cycle Analyses. For flow microfluorometry (FMF), cells were stained with mithramycin and analyzed as described (17). Measurement of [^{3}H]thymidine incorporation into the acid-insoluble fraction and autoradiography were described by Martin *et al.* (18). Isolation of DNA was performed according to that of Morimoto *et al.* (19), except that the cells were prelabeled with [^{3}H]thymidine. After hydrolysis in 1 ml of 1 M perchloric acid, 0.4 ml samples were taken for measurement of radioactivity and optical absorbance at 266 nm. Cell number was determined by use of a Coulter counter. Protein concentrations were measured by the method of Lowry *et al.* (20).

Measurement of Insulin Content. Insulin content was assayed by using an immunoassay kit from Schwarz/Mann, Becton Dickinson and Co.

RESULTS

The presence of serum in the culture medium has been one of the difficulties in defining an insulin effect. For the present study, serum starvation was carried out at least 12 hr prior to the addition of insulin or serum, as described in *Materials and Methods*.

Results of dose response to insulin shown in Fig. 1 indicated that the cell number increased and reached a plateau at a

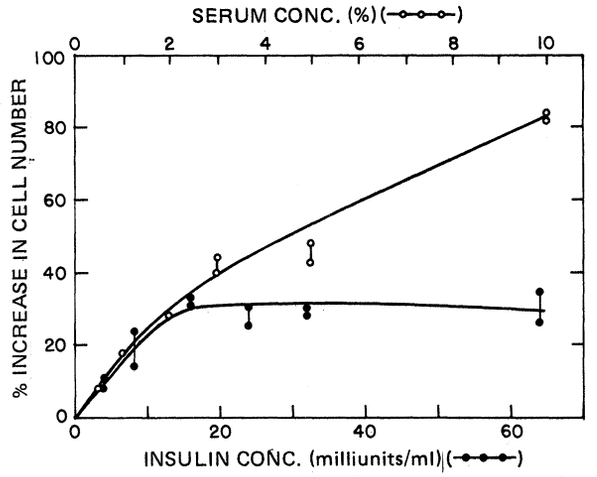

FIG. 1. Dose response of cell growth to serum and insulin. Secondary chick fibroblasts were prepared as described in *Materials and Methods*. After serum starvation, the indicated amounts of insulin and serum were added to cultures in fresh Medium 199. Thirty hours later cells were counted. The result is expressed as the percentage increase in cell number over the control cultures: insulin (●—●); serum (O—O).

concentration of 16 milliunits of insulin per ml of medium. A comparable increase in cell number occurred with 3% chick serum. Thus, 16 milliunits of insulin per ml and 3% chick serum were chosen for further comparative studies and are reported here.

Insulin Stability under Culture Conditions. It is important to know the stability of insulin under the experimental conditions used before its effect can be studied. A competitive radioimmunoassay for insulin was performed on media from plates with or without cells and with either 3% chick serum or with 16 milliunits of insulin per ml. There was no detectable amount of insulin as measured by this assay in medium containing chick serum. In the medium containing insulin, with or without cells, the level dropped at 4 hr to 50% and at 26 hr to 20–30% of the original value.

Cell Cycle Sequence Following Insulin and Serum Addition. There is considerable evidence that, once resting cells are stimulated by serum for 7–10 hr, they are able to traverse the complete cell cycle. Whether or not the same is true for insulin stimulation has been the subject of much argument. To investigate the movement of insulin- and serum-stimulated cells through the cell cycle, we used FMF. The distribution pattern of cells in G_1, S, and G_2 + M phases of the cell cycle based on the cell DNA content are demonstrated in Fig. 2. In all the control histograms, there was a peak of G_2 + M cells representing from 5 to 10% of the total population. Additional movement of cells out of G_1 was evident only in cultures stimulated with insulin or serum. FMF patterns indicated that insulin-stimulated cells began entering S before serum-stimulated cells. However, the former appeared to move through S and G_2 + M more slowly and synchronously than the latter. The insulin-stimulated cells began accumulating in G_2 + M starting at about 14 hr; whereas, the serum-stimulated cells never really accumulated in this phase of the cell cycle but moved on through mitosis into G_1. This observation is consistent with the increase in cell number occurring in serum-stimulated cultures before the increase seen with insulin stimulation, as will be discussed later.

To determine the total number of cells in each culture that had traversed S and G_2 by 22 hr, mitosis was prevented by the presence of 10^{-7} M colcemid. The proportions of the population

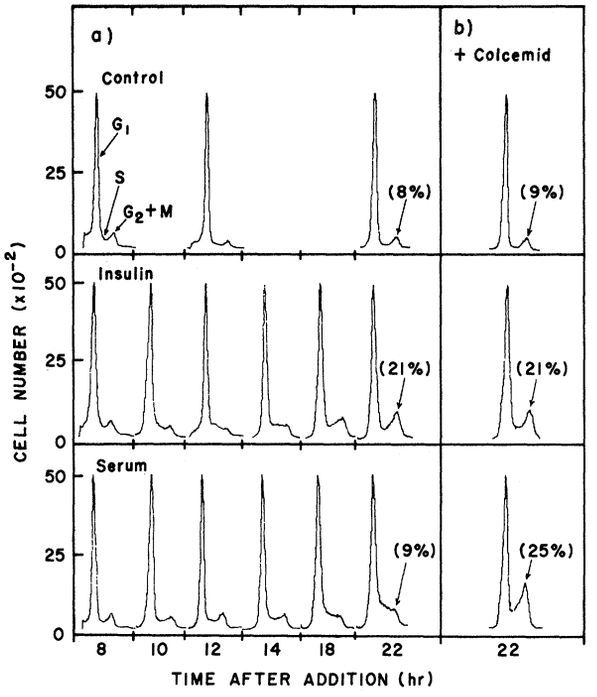

FIG. 2. Cell cycle movement after insulin or serum addition. The cells were harvested and analyzed by FMF. The results are presented as cell number versus the DNA content (arbitrary units) for each time point. (a) Serum starved cultures were provided with fresh Medium 199 with or without insulin or chick serum. (b) Colcemid added at concentration of 0.1 μM to parallel set of plates at 3 hr after medium change. Cells were harvested at 22 hr. The numbers in parentheses indicate the total cells in G_2 + M in each case.

in G_2 + M in the presence and absence of colcemid were comparable in control cultures. This was also true for insulin-treated cultures, indicating that in both cases few if any cells had finished mitosis by 22 hr. In serum-treated cultures, however, there were more cells in G_2 + M in the presence of colcemid than in its absence (in this experiment 25% versus 9%). A large proportion of cells stimulated by serum had already gone through mitosis by 22 hr. FMF results were compared with the conventional procedures used for growth measurement. These were carried out in detail and are discussed below.

Kinetics of [^{3}H]Thymidine Incorporation into Acid-Precipitable Fraction. To detect the rate of DNA synthesis, cultures were pulsed with [^{3}H]thymidine for 1 hr at various times after the addition of insulin and serum. Fig. 3 shows the kinetics of [^{3}H]thymidine incorporation into the acid-insoluble fraction. A simple change of medium caused a slight increase in DNA synthesis in control cultures. Incorporation of [^{3}H]thymidine increased during 2–3 hr after insulin addition and reached a maximal level at 8–10 hr. The rate of incorporation decreased thereafter. The onset of response to serum occurred later and the maximal level was lower than the maximal level with insulin.

Measurement of Total Content and Specific Radioactivity of DNA. The total DNA content was measured after the addition of either insulin or serum (Fig. 4). An appreciable increase in DNA content occurred 11 hr after insulin addition. Because some cell detachment occurs in the absence of serum, the total DNA level per plate is usually underestimated after insulin addition, especially at early time points. In addition, the actual increase in total DNA is initially too small to measure under these conditions. A measurement of the specific radio-

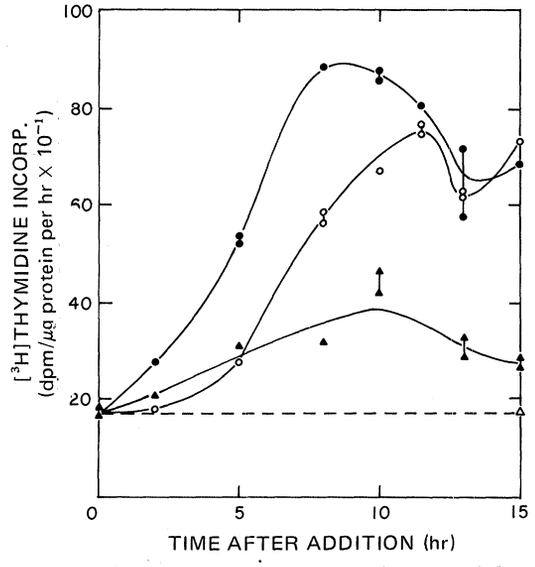

FIG. 3. Insulin and serum effect on the kinetics of thymidine incorporation into acid-precipitable fraction. After serum starvation, insulin and serum were added at concentrations of 16 milliunits/ml and 3%, respectively, at various times. [^{3}H]Thymidine incorporation was carried out all at once by labeling cultures for 1 hr with 1 ml of Medium 199 containing [^{3}H]thymidine, 2 μCi/ml (specific activity of 20 Ci/mmol) (20). The counting efficiency was 30%, with a background of 20 cpm. Insulin (●—●); serum (○—○); control without medium change (△ – – △); control with medium change (▲—▲).

activity of DNA after the addition of serum or insulin was therefore undertaken. Fig. 5 shows the specific radioactivities of the DNA isolated at 5, 11, and 15 hr after growth stimulation. The data agree with that of [^{3}H]thymidine incorporation into acid-insoluble material. A higher rate of incorporation into acid-insoluble material corresponded to the higher specific radioactivity of DNA in insulin-treated culture versus serum-treated culture. It is possible that, in addition to stimulation of DNA synthesis, insulin also causes an increase in the available radioactive thymidine pool by either increasing the uptake or suppressing the endogenous synthesis of thymidine. This could explain the small discrepancy between the radioactivity in acid-precipitable material and the specific radioactivity of DNA

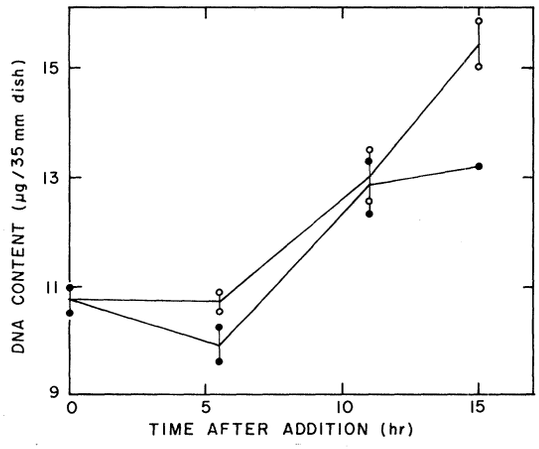

FIG. 4. Kinetics of increase in total DNA in the presence of insulin or serum. Fresh Medium 199 either alone or with insulin or serum was applied to serum-starved cultures. After various time intervals, [^{3}H]thymidine 1 μCi/ml (10 mCi/mmol), was added for 4 hr. Isolation of DNA and measurement of specific radioactivity were described in *Materials and Methods*. Values are averaged from quadruple samples: insulin (●—●), 3% chick serum (○—○).

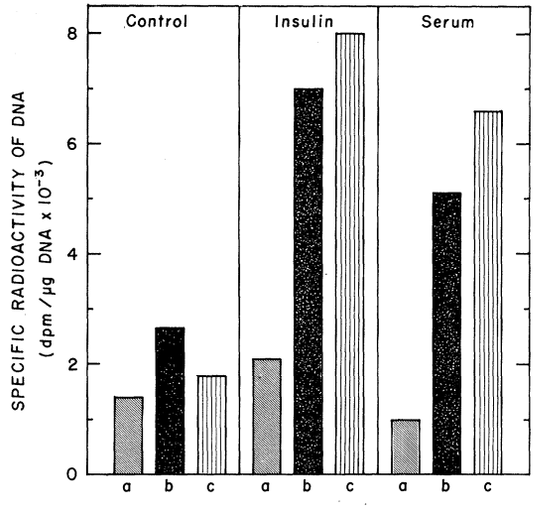

FIG. 5. Specific radioactivity of isolated DNA after the addition of insulin and serum. The specific radioactivities of DNA in same cultures as in Fig. 4 labeled from (a) 1.5–5.5 hr ▧; (b) 5–11 hr ■; and (c) 11–15 hr ▥.

on the one hand, and total increase in DNA synthesis on the other hand, especially for the early time points.

To bring the various elements of our measurements together and determine their correlation with the increase in cell number, the experiment shown in Fig. 6 was performed. Fig. 6a shows a 1 hr pulse of [^{3}H]thymidine incorporation into the acid-insoluble fraction, which, as shown above, correlates with the actual DNA synthesis in our system, as indicated in Figs. 4 and 5. While after 27 hr, cells in serum continue to synthesize DNA at a slower rate, insulin-treated cultures have gone through one synchronous wave of DNA synthesis. Fig. 6b shows the approximate number of cells in S and G_2 + M as a function of time calculated from FMF studies. Fig. 6c shows the percent of labeled nuclei as measured by autoradiography after continuous labeling with [^{3}H]thymidine. The data are consistent with the idea that cells in insulin enter S earlier than those that are serum-treated, and that the cells show prolonged, but synchronous, S and G_2 + M phases. The continuous increase in the percentage of labeled nuclei in serum-treated cultures then is due in part to early division of previously labeled cells. Fig. 6d depicts the increase in cell number which is consistent with the above picture. As can be seen in control cultures, despite some DNA synthesis, the number of cells decreases due to their detachment in the absence of serum. Insulin-treated cells remain constant probably due to a steady-state situation created by cell detachment and replacement. They finally go through mitosis after 27 hr. Cells in serum begin to increase in number early and continue to do so during the course of the experiment.

DISCUSSION

A primary or secondary culture of chick embryo fibroblasts has often been used as a model for studying growth regulation. The growth response of these cells to serum and hormones, such as insulin, has been quantified and compared in various laboratories. The answer to the question of whether or not insulin, like serum, is capable of inducing growth has not been a simple one to find. However, there is no reason to expect that a single compound such as insulin should elicit the same response from the cells as that of a complex mixture-like serum. It is possible that the growth-stimulating activity of insulin on chick cells is nonspecific and subject to the particular culture conditions used. On the other hand, the recent demonstration of the existence of insulin receptors on the surface of the chick embryo fibro-

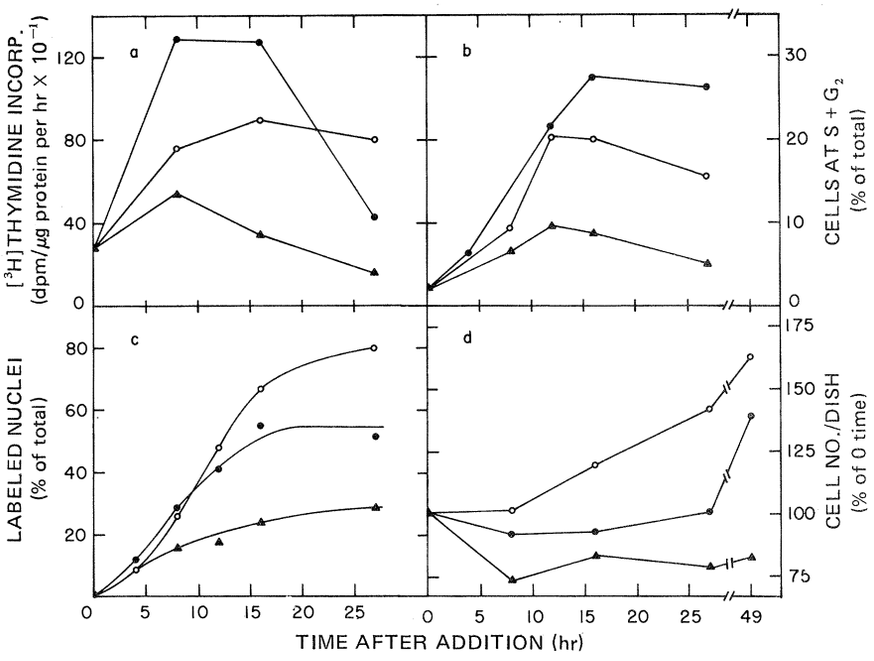

FIG. 6. The kinetics of four growth parameters after the addition of insulin or serum. The techniques for [³H]thymidine incorporation (Fig. 6a), FMF analysis (Fig. 6b), autoradiography (Fig. 6c), and for cell counting (Fig. 6d) were described in *Materials and Methods*. The [³H]thymidine added was at 1 μCi/ml (specific activity was 20 Ci/mmol; Fig. 6a) and 1 μCi/ml (specific activity was 5 Ci/mmol; Fig. 6c). This proportions of cells at S and G_2 + M phases shown in Fig. 6b were obtained by integration of FMF patterns. Fresh medium (▲—▲), insulin (●—●), and serum (○—○).

blasts (21) indicates that the action of insulin on these cells indeed may be the result of hormone-receptor interaction.

There seems to be little agreement in the literature as to the means by which "growth" or "mitogenicity" should be measured. The measurement of acid-precipitable radioactivity after a 1 hr pulse with [³H]thymidine has been the most popular procedure for DNA measurement. However, because this procedure is dependent on both the rate of uptake of thymidine and its incorporation into DNA, it can be used only as a qualitative estimate of DNA synthesis. In addition, the sampling time is of critical importance. Short sampling times after insulin addition would tend to exaggerate the degree of stimulation of DNA synthesis; sampling times between 15 and 30 hr would miss the effect entirely due to the synchronous stimulation of DNA synthesis and delayed mitosis. Measurement of the specific radioactivity of DNA isolated from cells at various times after stimulation gives a more accurate rate of [³H]thymidine incorporation into DNA, but is still subject to the variation in the specific radioactivity of the thymidine pool. Measurement of total DNA per dish suffers from the insensitivity of DNA analysis technique, and is also subject to the variation caused by cell detachment. The cell population stimulated to synthesize DNA can be measured by autoradiography, if the cells are continuously labeled with [³H]thymidine. This technique is very sensitive, gives some information about the synchrony of the stimulated population, but may give false positives due to the existence of DNA repair. In addition, the procedure is tedious and would tend to exaggerate the percent increase in labeled nuclei after serum addition because of rapid cell division unless mitosis is inhibited.

In this report, we chose to compare the results obtained by all these techniques and correlated them with flow microfluorometric analysis, a technique that measures the actual progression of the cells through the cell cycle.

The picture that emerges of insulin action on confluent culture of chick fibroblasts is as follows: 16 milliunits of insulin per ml of culture medium, which is close to physiological insulin level, stimulates 30–50% of the cells to enter S after 2–3 hr. The cells move rather synchronously into G_2 with a mean S residence of 6–8 hr. The cells eventually divide, after a mean G_2 + M residence of 12–16 hr. By any definition of "mitogenicity", therefore, insulin is mitogenic for a significant proportion of cell population under our culture conditions. It can further be concluded that insulin acts during some part of the G_1 phase of the cell cycle. In addition, the kinetics of the insulin response explains some of the contradictory results reported in the literature (4, 10, 12). Yet it is obvious from Fig. 1 that increased levels of insulin above 16 milliunits/ml cannot increase the porportion of cells that are induced to divide. Whether the division of insulin-stimulated cells is an unavoidable, albeit slow, result of DNA synthesis stimulation or whether factors necessary for the completion of G_2 and mitosis are slowly provided by cells themselves is not clear.

When this pattern of insulin action is compared to that of 3% chick serum, the following differences and similarities are observed: the total cell population that is stimulated to enter S and G_2 is comparable initially (within 15 hr). However, serum-stimulated cells enter S later than insulin-stimulated cells, and have a shorter mean residence in S and G_2. They therefore divide earlier than insulin-stimulated cells. In addition, increasing the serum levels leads to increasing the proportion of cells that can divide as shown in Fig. 1.

The question arises as to why insulin stimulates only part of the cell population. The possibility that there are two distinct populations in chick embryo fibroblasts, only one of which responds to insulin, cannot be entirely ruled out at this time. This, however, is not a very likely possibility. An additional question is why insulin does not stimulate DNA synthesis in certain mammalian cell lines. Rudland *et al.* (13) showed that insulin alone is incapable of stimulating DNA synthesis in Balb 3T3 cells (our unpublished data confirm this). They suggested that insulin is just a permissive factor for other growth-stimulating

agents, e.g., fibroblast growth factor, rather than acting as a growth stimulant. Similar conclusions were drawn for insulin versus prolactin activity (22). However, it was demonstrated that the action of insulin on DNA synthesis is not confined to the avian system alone. Insulin clearly induces a significant amount of DNA synthesis in primary organ culture of mammary gland (23). Therefore, either a fundamental difference between cell lines and primary cultures exists with regard to insulin action, or some attempt to integrate the two types of cultured cells must be undertaken, since both are used as models for growth regulation. We propose a hypothetical model which can be tested experimentally.

One obvious difference between the cell lines and primary cultures is the "tightness" of density-dependent inhibition of growth. While cell lines rarely form multiple layers, except in the presence of very high serum concentrations, primary cultures can form multilayers even under low serum conditions (24, 25). We would therefore like to propose that primary cultures, whether avian or mammalian, being closer to the *in vivo* state, would respond to a three-dimensional growth control, while tightly density-inhibited cell lines are more strictly two-dimensional. In other words, the cells at the top layers of primary cultures continue to proliferate slowly while the cells at the bottom layers are analogous to those in a tissue and have more stringent requirements for growth induction. The cells in the top layers of primary cultures would divide slowly, even without stimuli at high cell densities, as control culture in Figs. 2, 3, 5, and 6 indicate. However, at no time would more than 5–10% of the population go through mitosis under these conditions. Insulin alone would stimulate only those additional cells that have not reached complete three-dimensional arrest (about 30–50% of the population under our conditions). Other factors (or merely a higher concentration of other factors), which are present in serum, would be required to "turn on" cell lines or cells at the bottom layer of primary cultures. For these cells, then, insulin alone is not sufficient to induce DNA synthesis.

In contrast to Balb 3T3 cells, where no stimulation of DNA synthesis is observed with insulin, Swiss 3T3 cells show a small, but measurable, increase in labeled nuclei from 0.2 to 4% (26, 27). Interestingly, we have observed in this laboratory that the growth of Swiss 3T3 cells is less stringently controlled than that of Balb 3T3 cells. We propose that there is a direct relation between the "tightness" of density control and the magnitude of insulin effect.

The question of whether insulin can stimulate only one round of DNA synthesis while serum can induce more cannot be answered from our data. The instability of insulin under the culture conditions should be taken into consideration when its long-term effects are examined. If our hypothesis is correct, fresh insulin should still be capable of stimulating another round of DNA synthesis in those cells which have not reached the complete three-dimensional growth arrest. Our preliminary data indicate that this may be the case.

The final question, how insulin exerts its effect on growth of some cells, remains unanswered. The binding of insulin to the plasma membrane of liver and fat cells, and human and chick-embryo fibroblasts, has been demonstrated. Some studies have indicated that the receptor site may be the same for insulin and other growth-stimulating hormones such as somatomedin or multiplication stimulating factor (28). It would be important to know whether cell lines also contain insulin receptors despite their lack of response to growth induction. Furthermore, whether binding alone is sufficient to trigger DNA synthesis or whether additional metabolic changes are needed, remains to be seen. Alternatively, the ability of insulin to stimulate growth may be due to an entirely different aspect of its structure, such as the proteolytic activity reported to be associated with this hormone (29).

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